ROYALTY RESEARCH FUND ■ RRF SCHOLAR APPLICATION (RRF-1) For PI

Name F/M/L	Steven Beyer Roberts	Rank	Assistant Professor
EID	859 –009 –248	Dates of —	
Department	School of Aquatic and Fishery Sciences	Initial Appt	December 2006
Box Number	355020	to Faculty	December 2006
Phone	(206)600-4495	to Present Rank	December 2006
E-mail	sr320@u.washington.edu		(Enter dates as month-year, i.e. March-2002)

Project Title: Proteomic response of Pacific oysters to environmental stress

(Please limit to 120 characters)

Application for RRF Scholar ☐ YES ☑ NO ☞ *If YES, use an additional page to document teaching load. List quarter, course no., title and credits*

Research is best classified as *(check only ONE)*

- ☐ Arts or Humanities
- ☐ Physical Sciences
- ☐ Engineering
- ☑ Basic Biological or Biomedical Sciences
- ☐ Social or Behavioral Sciences
- ☐ Clinical Biomedical Science

Abstract of Proposed Research *(limit to space provided)*

The Pacific oyster is a critical component of our local waters with significant ecological and economic standing. When oysters are exposed to stressors such as poor water quality, harmful compounds, or pathogens, the organism's normal physiological processes become compromised. In most instances this leads to decreased survival with detrimental effects on larger systems. In shellfish, information is lacking regarding the direct and indirect response to stress at the proteomic, or global protein level. The goal of this research is to examine the stress response in oysters using a proteomic approach. The specific objectives of this project are to 1) identify proteins differentially expressed in response to temperature change, 2) identify proteins differentially expressed in response to pathogen exposure, and 3) examine corresponding gene expression levels. While proteins are ultimately the molecules mediating the stress response, it has only recently become technically feasible to take such an approach. The ability to accurately assess physiological stress in oysters will not only provide valuable insight into the interaction of stress and normal physiological functions such as growth, reproduction, and immune function, but will also assist in identifying subtle environmental change in our aquatic ecosystems.

If answer to any of the following is YES, please explain on an additional page. Where appropriate, include indications of approval. *Submission to the appropriate approval committees is not necessary until after awards are made.*

YES	NO		
☐	☑	1.	Will space not now available be required?
☐	☑	2.	Will the proposed project be conducted off campus?
☑	☐	3.	Does the proposal involve radionuclides ☐; x-ray ☐; pathogenic organisms ☑; human or animal blood ☐; cells, tissues, body fluids ☐; recombinant DNA ☑; chemical carcinogens-mutagens-teratogens ☐; diving ☐?
☐	☑	4.	Does the proposal involve the use of human subjects?
☑	☐	5.	Does the proposal involve the use of animals?
☑	☐	6.	Is more than one department, school, or college involved in the proposal?
☑	☐	7.	Is an interdisciplinary facility or service center involved?
☐	☑	8.	Are researchers or facilities at another university, government agency, or other institution involved?
☐	☑	9.	Will restriction on information or security classification be required?
☐	☑	10.	Are any special resources required, e.g., expanded library services or materials, purchase of special services?
☐	☑	11.	Does the proposal represent a continuation of work previously or currently supported by other funding?
☑	☐	12.	Have you previously submitted a proposal to the RRF? If so, when?
☑	☐	13.	Is this a resubmission of a previously unfunded proposal to the RRF?

Budget Summary *(Round to nearest dollar)*

Salaries and Wages	01	191278
Contract Personal Services	02	
Other Contractual Services	03	6500
Travel	04	300
Supplies and Materials	05	4000
Equipment	06	
Retirement and Benefits	07	3412
Operating Fee/Tuition	08	6222
TOTAL BUDGET		39562

Signatures (Submit a separate RRF form for the PI and each co-PI)

Proposed by _____
Principal Investigator Date

By signing, the PI agrees to abide by all RRF policies and procedures and accepts responsibility for any budget deficits.

Approved _____
Chair/Director Date

Approved _____
Dean of the College Date

By signing, the Dean confirms that the applicant holds an eligible faculty rank, or has PI status.

Explanations for YES answers on RRF form

3. Live cultures of *Vibrio tubiashii* and *Vibrio parahaemolyticus* will be used for oyster exposure experiments. Both strains are not pathogenic to humans. Routine gene cloning techniques involving recombinant DNA technology will be used.

5. This project involves a mollusk, the Pacific oyster (*Crassostrea gigas*).

6. Shotgun proteomics will be carried out in the Mass Spectrometry Center at the University of Washington's School of Pharmacy. The Director of the Center is Dr. Dave Goodlett, faculty in the Department of Medicinal Chemistry.

7. The Mass Spectrometry Center is an interdisciplinary facility

12. This is a resubmission of a proposal I submitted in Fall 2008.

13. This is a resubmission.

Reviewer Responses

This is the second resubmission of the enclosed Royalty Research Fund proposal. The first set of reviewer responses included suggestions such as elaborating on oyster's "*connection to society*", highlighting shellfish as great environmental indicators, and adding explanations of how non-ideal genomic data would impact results. In the first resubmission these and other items were addressed.

For this most recent submission, two reviewer's comments were provided. Each reviewer's comments are addressed below.

Reviewer #1

This reviewer's comments were primarily descriptive in nature. The reviewer indicated that although identification of proteins using mass spectrometry was "*explained only briefly, such methods are well established for species are whose genomes are sufficiently well characterized*". In this revision I provided more detail about the techniques used for protein identification. The reviewer goes on to state the PI "*is well situated to extend his results into the use of any specific signals he identifies as potential indicators of stress, with implications for their use in commercial management*".

Reviewer #2

Comments from Reviewer #2 were very positive with statements such as "*the author demonstrates sufficient technical competence in the proposed field, uses appropriate methods to gain novel insights...and is likely to make significant contributions to the field*." While indicating the two-dimensional electrophoresis was appropriate and acknowledging a previous reviewer stated "*2D PAGEMS analysis the best choice technically*", Reviewer #2 did go on to suggest the possible use of shotgun based LC-MS/MS. In other words, instead of using 2D gel electrophoresis to identify differentially expressed proteins, simply identify all proteins and quantify levels between samples using solely LC- MS/MS. I discussed this suggestion with the Director of the Mass Spectrometry Center at the University of Washington's School of Pharmacy, Dr. Dave Goodlett. We came to the conclusion that given the potential inconsistencies in electrophoresis and added benefits of having increased proteome coverage, taking a shotgun approach using gas-fractionation to separate samples based on the optimal m/z ranges would be appropriate. The proposal has thus been modified to reflect this. More details about the preliminary oyster protein analysis were also included in this revision. Reviewer #2 "*recommend[ed] this proposal for the highest priority of support*".

Proteomic response of Pacific oysters to environmental stress

All organisms are exposed to stimuli that disrupt normal physiological processes and the maintenance of homeostasis. This can be referred to as stress. These disruptions frequently compromise processes involved in normal immune function, growth, reproduction, and sometimes survival. Such stimuli or stressors can include variations in environmental parameters, toxic compounds, or exposure to pathogens. Often stressors will not only affect population dynamics but could threaten entire ecosystems either directly or indirectly by altering specific members of the community. Negative consequences on growth, reproduction, and survival to species of commercial importance such as shellfish, would additionally have significant economic impact.

The research proposed here focuses on the environmental stress response of the Pacific oyster (*Crassostrea gigas*), an important natural resource in the Pacific Northwest having substantial economic and ecological importance. Pacific oysters are the key cultured shellfish species in the US with production estimated at 40,601 tonnes (WRAC, 2004; FAO 2006). Washington State produces approximately 87% of US Pacific oysters and California produces most of the rest. In addition to harvest-sized product, 35 million Pacific oyster seed were produced in 1995 (WRAC 2004) and production has increased since that time. Oyster farming is estimated to contribute over $100 million annually to the region's economy (WRAC, 2004).

As benthic filter-feeders, oysters play an important role in estuarine food webs and contribute to the removal of excess organics, nutrients, and particulates (Newell et al. 1999; Rice 1999; Rice et al. 1999, Officer et al. 1982). Nutrients such as nitrogen and phosphorus that are not incorporated into oyster tissue are excreted and can then be utilized by keystone plants such as eelgrass (Newell 2004). Plants also benefit from increased sunlight associated with the oyster's ability to improve water quality via particulate removal (Newell et al. 2002). Oysters commonly grow, and are farmed, in aggregations that have been shown to enhance biodiversity (Ferraro and Cole 2007). Such structures are integral not only for larval oysters, but for other organisms such as worms, snails, crabs, fish and birds that utilize the structures as habitat. Shellfish such as oysters are also carbon fixers, incorporating carbon into their shell thereby helping to reduce carbon dioxide levels (Newell 2004).

Mollusks have become one of the most important and frequently used experimental models for environmental research (review: Rittschoff and McClellan-Green, 2005). In addition, mollusks have been valuable as sentinel monitors of contaminants. The attributes that make shellfish exceptional as environmental monitors are 1) they are common; 2) widely distributed; 3) immobile with high site fidelity; 4) reasonably resistant to contaminants; and 5) continuously filter the water that they live in. Oysters are commonly used as estuarine and marine indicator species as they are easily obtained, likely to survive test conditions, and accumulate the contaminants of interest to concentrations proportional to ambient waters (Phillips and Rainbow, 1993).

The goal of this proposed project is to carry-out the first proteomic-level study of the stress response in the Pacific oyster. Specifically, the physiological response of oysters to two environmental stressors will be examined. The remainder of this section first provides a brief review of the stress response in shellfish. Following a discussion of specific stressors and what is referred to as the general stress response, the end of this section summarizes the lack of knowledge we have concerning the proteomic response. It is only now with the convergence of advances in mass spectrometry and development of large genomic datasets that we are able to successfully carry out the research objectives of the current project. Completion of this research will provide insightful data on the invertebrate stress response and these data will lay the foundation for a variety of other large-scale integrated projects, including those focused on i) developing tools to assist the aquaculture industry, ii) better understanding invertebrate physiology to improve environmental conditions for species survival and iii) application of environmental proteomics to better evaluate anthropogenic impacts on aquatic ecosystems.

Background: Temperature Stress Marine invertebrates living in the intertidal are faced with numerous stressors including predators, wave action, pollution, desiccation, and fluctuations in temperature. In a majority of instances these environmental stressors are not lethal but can impact other vital physiological functions. There has been significant research on the impacts of environmental stress on

shellfish in part due to the fact that shellfish are excellent bio-indicators. Temperature stress is a common stressor that is examined and is of particular interest given the predictions of large-scale climate variation. While not the only response, one of the responses to temperature stress is expression of Heat Shock Proteins (HSPs). Heat-shock proteins are a small family of molecular chaperones that (despite their name) do not include all proteins regulated by temperature and environmental stress. Furthermore some HSPs are constitutively expressed and temperature does not alter expression. In both stress and unstressed cells, HSPs and other proteins are involved in folding, assembly, localization, secretion, regulation, degradation, and removal of other proteins (for review see Feder and Hofmann 1999). The need for these tasks is often higher when an organism is exposed to a stressor since this exposure commonly results in proteins assuming non-native (e.g., denatured) conformations. When routine cellular "housekeeping" processes are not carried out properly, there are usually negative effects on other physiological processes as the denatured proteins interact non-specifically with other molecules. Numerous studies have examined temperature induced HSP expression in invertebrates (e.g. Clegg et al 1998; Krebs and Feder, 1997; Singh and Lakhotia, 2000; Spees et al, 2002).

Background: Disease Stress When shellfish are exposed to a pathogen (e.g., bacteria, virus, eukaryotic parasite) the response is often specific to the mode of action of the pathogen. To date, most of this research effort has been at the transcript level or focused on a small family of proteins, primarily selected based on their putative function in other taxa. There are a variety of molecules involved in the innate immune response to pathogens including, antimicrobial peptides (Gueguen et al, 2006; Stensvåg et al, 2008), proteases and protease inhibitors (Zhu et al, 2006), lysozymes (Matsumoto et al, 2006; Xue et al, 2007) and lectins (Kang et al, 2006; Kim et al, 2006; Gourdine at al, 2007). My lab has been involved in characterizing the immune response of the Eastern oyster (*C. virginica*) exposed to the pathogen *Perkinsus marinus (*Roberts 2008*)*. In addition to some of the mechanisms listed above, we also have seen evidence of apoptotic pathway activation which has also been reported by other researchers (Sunila and LaBanca, 2003). More recently, our lab has begun to characterize the immune response of *C. gigas* exposed to pathogenic *Vibrio* species at the transcriptomic (mRNA) level. We have characterized the first interleukin gene from an invertebrate (Roberts et al, 2008a), and recently characterized a suite of genes originally identified from a cDNA library we constructed from hemocytes, the primary immune cells in oysters (Roberts et al 2008b). It is not known whether corresponding protein levels also change. Figure 1 shows gene expression levels from oyster gill tissue taken from oysters exposed to *Vibrio* for 24 hours and controls.

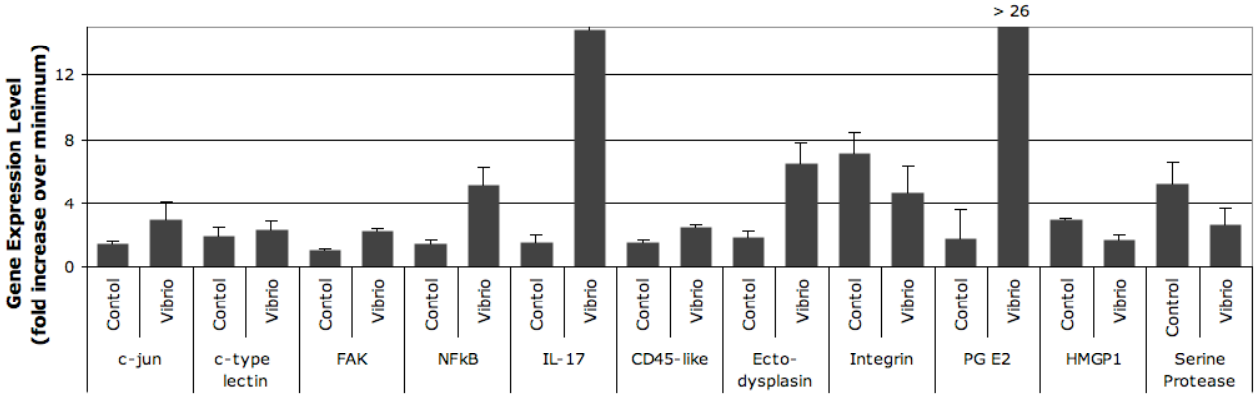

Figure 1. Mean gene expression levels in gill tissue of oysters (*C. gigas*) exposed to *Vibrio* species for 24 hours and controls. Modified from Roberts et al 2008b. Protein expression levels of these products are not known.

Background: General Stress Response - Vertebrates In vertebrates, the two primary compounds produced during almost all forms of stress are glucocorticoids (e.g., cortisol) and catecholamines (e.g., adrenaline), and are considered part of the general stress response in vertebrates (for review see Norris 1996). There are two primary pathways regulating the response, the hypothalamic-pituitary-steroidogenic cell axis and the hypothalamic/sympathetic nervous system/chromaffin cell axis,

respectively. Release of glucocorticoids from steroidogenic tissue (e.g., adrenal gland) is triggered first by a stressor initiating corticotropin-releasing hormone from the hypothalamus that signals pituitary adrenocorticotropic hormone (ACTH) secretion (a pro-opiomelanocortin (POMC)-derived peptide). In the other pathway, the hypothalamus controls the release of catecholamines by chromaffin cells through ACTH and cholinergic fibers of the sympathetic nervous system. The means by which an organism's system reacts to stress has been described as the general adaptation syndrome (GAS) and is divided into 3 stages. The first stage is known as the "alarm reaction." At this stage there is increased secretion of glucocorticoids and sympathetic nervous system stimulation. Stage 2, or the stage of resistance, is marked by the tolerance or adaptation to stressful stimuli and is characterized by a prolonged and increased secretion of glucocorticoids. In the third stage, or stage of exhaustion, the ability of the animal to function is severely impaired and usually leads to death.

Background: General Stress Response – Invertebrates Knowledge about the stress response in invertebrates is limited, and it is not known if a general response that is stimulated by all stressors exists. However, there is evidence to suggest that some of the components of the glucocorticoid and catecholaminergic pathways are present in invertebrates. A corticotropin-releasing hormone-like molecule has been reported in an annelid (Remy et al, 1982), an insect (Verhaert et al, 1984), and the mollusk, *Planorbarius corneus* (Ottaviani et al, 1990). ACTH-like molecules have also been reported in several invertebrates including bivalve mollusks (Franchini and Ottaviani, 1994; Smith et al, 1990). Catecholamines such as noradrenaline have been identified and measured in a variety of invertebrates (Ottaviani and Franceschi, 1996). In the Pacific oyster and in abalone, significant increases in noradrenaline and dopamine in the hemolymph have been reported during mechanical stress (Lacoste et al, 2001c; Malham et al, 2003). Just as with vertebrates, it has also been shown that ACTH stimulates the release of noradrenaline in bivalves (Lacoste et al, 2001b). Interestingly, Lacoste et. al. (2001a) have indicated that noradrenaline can induce expression of HSP70 in bivalve mollusks. While similar molecules have been identified analogous to the stress response in vertebrates, the fundamental difference is that the invertebrate system is simplified. There is no hypothalamus, pituitary, or adrenal gland in the oyster but rather the hemocytes are the primary cells that produce compounds involved in the stress response (e.g, ACTH) and are also the primary mediators of the cellular response to pathogens.

The Research Problem: Previous research has provided insight into stressor-specific responses and has suggested that certain general stress-related pathways and products are present in bivalves. The basis for this identification of molecules in the glucocorticoid and catecholaminergic pathways has been, in many cases inferential, using heterologous antibodies or probes to demonstrate immunohistochemical or *in situ* signals, respectively (e.g., Ottaviani et al, 1991; 1998; Goldfarb et al, 1989; Wootton et al, 1995). In addition, in all cases, research has been directed at a specific, predetermined pathway (e.g., catecholamines and HSP). Only a handful of proteins involved in the stress response have been absolutely identified by protein sequence analysis in bivalves or any invertebrate. It is essential at this point to obtain protein sequence data to completely validate the identity of components of the stress response. Without sequence identity we are guessing at the real players in these responses. ***More importantly, it is likely that there are invertebrate-specific protein level responses to stress that may be different than those observed in vertebrates and involve proteins that we are not currently investigating***. Thus, without taking a more global experimental approach, comprehensive identification of those pathways would be extremely difficult.

A Solution in Proteomics: Ultimately it is the proteins that carry out biological processes in an organism. Studying protein expression can directly inform researchers on physiological process and response to changes in environmental conditions. While advances in DNA sequencing technology allows for identifying changes in the transcriptome, this does not necessarily reflect corresponding protein level changes. For instance, mRNA transcripts may or may not be translated to proteins. In addition, a given set of conditions may result in the synthesis of a protein from pre-existing mRNA, or the release of protein from cellular stores. In order to examine global protein level changes proteins first have to be 1) separated, 2) cleaved into small pieces (~20 amino acids), 3) separated using liquid

chromatography, 4) sprayed into a mass spectrometer where the intensity and m/z (mass to charge ratio) of individual peptides and peptide fragments are measured. In this last step the small peptides originally loaded are broken again to smaller fragments. Given that almost all amino acids have unique masses, the individual amino acids from the original ~20 amino acid peptide can be determined. The bottleneck for non-model species then becomes the lack of genomic data sets to identify the short ~20 amino acid peptide. The strength of a proteomics approach depends on the genomic databases with which comparisons can be made. Obviously, the greater the number of usable (within open frames) sequences, and the closer the species are phylogenetically, the better the comparison will be. There have been many genomic sequencing projects providing sequence data for nonvertebrates. Some of these (e.g., *Drosophila*, *C. elegans*, *Ciona*, *Strongylocentrotus purpuratus* – sea urchin) will be helpful for proteomic approaches with mollusks. Several studies have illustrated the ability to use other species to identify proteomic data including research on halibut (Einarsdottir et al. 2007) and even the extinct *Tyranosaurus rex* (Smejkal and Schwitzer 2007).

However, what really facilitates using proteomics for studying the biology of the Pacific oyster is the recent dramatic increase in the number of expressed sequence tags available. As of February 2009, there are over 44,000 expressed sequences from the genus *Crassostrea* in GenBank, with a large proportion of them from cDNA libraries constructed from hemocytes or hemocyte-rich tissues including the >4,000 ESTs that we recently released (GenBank Accession numbers EW777381-EW779578; Roberts et al, 2008b). This is the same library used to initially identify *C. gigas* interleukin-17 (Roberts et al, 2008a) and other stress-related genes (Figure 1).

In order to determine the effectiveness of identifying proteins using this approach, a random protein spot was selected from a two-dimensional gel. In collaboration with the staff in the Mass Spectrometry Center at the University of Washington's School of Pharmacy we were successful in identifying the sample as primarily beta-actin using a customized protein database generated from translating all bivalve ESTs in 6 frames. Actual results can be viewed online [http://tinyurl.com/bxj8q2]. Furthermore, in addition to the sequences that are currently available, several concurrent sequencing efforts are underway including a Pacific oyster genome sequencing project by the Institute of Oceanology of Chinese Academy of Sciences, and the Beijing Genomics Institute, in collaboration with the international Oyster Genome Consortium (Liu and Rexroad, 2009). Given the increasing genomic datasets and our lab's successful identification of proteins from mass spectrometer results from oyster samples, we are confident that small peptides analyzed as part of the proposed project will be identified.

B. OBJECTIVES

The overall goal of the proposed project is to characterize proteins involved in the stress response in the Pacific oyster. We acknowledge the stress response can be (and has been) studied extensively at the transcript level. While the transcriptomic approach does provide accurate stress indicators, it does not provide the complete biological picture. The specific research objectives developed to accomplish this goal are to:

1) Identify proteins that are differentially expressed in response to temperature stress
2) Identify proteins that are differentially expressed in response to *Vibrio* exposure
3) Examine relationship of protein and gene expression for select targets

Completion of these research objectives will allow us to begin to test several hypotheses related to invertebrate stress including that a) multiple stressors will elicit a general stress response in oysters similar to what is observed in vertebrates, b) a cascade of events will occur in invertebrates that is analogous to the General Adaptation Syndrome characterized in vertebrates, c) stress-specific protein responses occur that will aid in predicting specific stressors in the environment and d) protein expression patterns will not always correspond to changes in gene expression level.

Development of these proteomic tools to examine oyster physiology will provide a platform to address numerous other critical issues in the future. From an environmental perspective, this approach will aid in development of invertebrates as environmental indicators. Protein expression patterns will complement current techniques and has the additional advantage of being

implemented in the future as a simplified assay (e.g. ELISA). Ultimately, by having an early warning system that there is a problem in our waterways, action could be initiated early on to remedy the problem. Furthermore, by taking a global approach we will be able to explore the evolutionary implications of stress on marine invertebrates. Environmental stressors ultimately select for individuals that are better adapted to survive. In our research we have observed this in oysters and abalone exposed to disease pressure. While an individual is better suited to survive a given stressor, secondary characteristics (e.g. related to growth and disease susceptibility) might also be under selection. Using a non-biased approach will certainly provide insight into the relationship of stress and other biological pathways that will assist in predicting long-term community impacts. For the aquaculture industry, the tools developed as part of this proposal could be applied to assist selection programs and provide information on the physiological condition of individuals that would help in developing better management practices. *Vibrio* species have had devastating effects on oyster hatcheries in the past few years. Since *Vibrio* is being used a stressor we will be able to directly assess successful host responses and have the opportunity to study environmental influences on pathogen virulence as part of a separate research project. For the current proposal we will be sacrificing individuals, however since hemocytes are the focus of the assays, non-lethal sampling could easily be adopted for commercial application.

C. PROCEDURE

Oysters: Oysters will be obtained from Taylor Shellfish Farms for the proposed experiments. We routinely maintain oysters from this source in our shellfish holding facility located in the Fisheries Sciences Building (FSH).

Temperature Stress: Following a ~2 week acclimation period in the FSH shellfish holding facility, oysters will be separated into two holding tanks with 15 oysters in each. In the control system water temperature will remain consistent (~18^{o}C). In the other system ("experimental") the water temperature will be raised to 28^{o}C. This temperature would not be lethal to oysters, but temperatures of 28^{o}C have previously been shown to stimulate changes in hemocytes (Hegaret et al, 2003). We have documented similar changes in hemocytes (i.e. phagocytic activity) and will use this along with gene expression analysis to corroborate protein changes with other stress indicators. Oysters will be sampled from tanks at 24 hours. Gill tissue will be sterilely removed and stored for later expression analysis. Individual samples will be pooled prior to proteomic analysis. The reason for selecting gill tissue is that gills are rich in hemocytes, the primary immune cells in shellfish. By using gill tissue we will also be able to see changes that might be exclusive to gill tissue (e.g., ion transport, membrane instability).

Pathogen Stress: Similar to the temperature stress experiments, oysters will be moved to separate systems following acclimation. Thirty oysters (15 control, 15 experimental) will be transferred to containers, with three liters of ~18°C sea water (consistent with main system). Challenges will be conducted with a mix of two species of bacteria: *Vibrio tubiashii* and *Vibrio parahaemolyticus*. Approximately 2.05×10^{11} bacteria will be used for the challenges. Briefly, the cultures will be centrifuged at 4000 RPM in a Sorvall ST-H750 rotor for 30 minutes at 4°C to pellet the bacteria. The supernatant will be removed and bacteria will be resuspended in 100 mL of sea water. This suspension will be added to the experimental system. The other container of 15 oysters will receive 100 mL of sea water. Oysters will be sampled from tanks at 24 hours. These are the same cultures (and protocol) that we use for examining gene expression in oysters (Figure 1). Gill tissue will be sterilely removed and stored for later expression analysis. Individual samples will be pooled prior to proteomic analysis. *Vibrio* samples will also be archived to study virulence and pathogen physiology as part of a separate research effort.

Shotgun Proteomic Profiling: The basic concepts of how mass spectrometry is used to identify protein samples were previously discussed (top of page 4). For the initial separation (step 1), one method commonly used to separate proteins is gel electrophoresis. In this case, bands or "spots" differentially expressed across gels (treatments) are excised and subjected to tandem mass spectrometric analysis. In fact this is how we have confirmed the ability to identify oyster proteins using mass spectrometric

analysis. However, based on the suggestion of a proposal reviewer and our own experience with inconsistencies of gel electrophoresis, for the current proposal we will submit entire protein samples to the Mass Spectrometry Center where the mass spectrometer will be used for initial separation via gas-phase fractionation. Recently, personnel from the Center published a manuscript demonstrating the procedure of iterative interrogation by a capillary liquid chromatography coupled to the nano-spray source of a tandem hybrid mass spectrometer (LC-MS/MS) with prior fractionation can effectively characterize the proteome without the need for gel separation (Scherl et al, 2008). Performing this analysis eliminates electrophoretic artifacts and provides a more complete picture of the proteome. The method is still able to identify differentially expressed proteins as amount of individual peptides are quantified in each mass spectrum, which is simply a plot of *intensity* vs mass-to-charge ratio (*m/z*). Initially, samples will be used to construct ion maps so that optimal *m/z* ranges can be determined. Then, in order to obtain more complete proteome coverage, each sample will be analyzed at least four times, each time at a different *m/z* range. This will insure highly expressed protein (i.e. albumin) will not mask proteins expressed at lower levels and provide greater statistical power to compare expression across treatments. Approximately ten LC-MS/MS analysis runs will be conducted from the temperature and pathogen stress experiments (5 treatment / 5 control). An additional five analyses are budgeted to allow for further analysis. The probable identity of the proteins will be determined by matching of amino acid sequence data obtained from LC-MS/MS analysis (see top of page 4 for explanation) against *in silico* processing of genomic data using ProteinProphet analysis software – the same procedures used for analysis of our preliminary experiments.

Gene Expression Analysis: During the end of the project we will begin to determine correlations (if any) of differentially expressed proteins with coding transcripts. If the genes are not present in sequence databases, efforts will be made to clone products using degenerative primers and/or rapid amplification of cDNA ends. We will perform gene expression analysis on approximately ten selected targets. Briefly, RNA will be isolated and reversed transcribed with the QuantiTect Reverse Transcription Kit (Qiagen), which also eliminates genomic DNA carry-over. Quantitative, real-time PCR reactions (25uL) will contain the following: 0.5uL cDNA, 0.04 uM forward/reverse primers, 1X Immomix Master Mix (Bioline) and 2uM SYTO13 (Invitrogen). An Opticon 2 thermocycler (Bio-Rad) will be used to quantify gene expression. Raw data will be analyzed using Real-Time PCR Miner Software (Zhao & Fernald, 2005).

D. TIME SCHEDULE

Temperature stress and associated shotgun proteomics will begin Summer 2009. Samples from both the temperature stress procedure and *Vibrio* challenge will be submitted to the UW Mass Spectrometry Center by early 2010. By Spring 2010 we plan on analyzing the results and carrying out complementary gene expression assays to examine the correlations of transcription and translation.

E. NEED FOR RFR SUPPORT

I firmly believe we are at a pivotal point where analysis of large-scale protein expression in *Crassostrea* is possible. Since we have just reached this point, demonstrating the benefits of this approach will provide unique opportunities to increase my competitiveness for subsequent funding. While I have studied environmental stress using a transcriptomic approach, taking a proteomic approach will be a new direction for my program. Not only will successful completion of the current proposal provide insightful data on the invertebrate stress response, these data will lay the groundwork for a variety of large-scale integrated projects. Examples include research focused on 1) understanding the basic mechanisms involved in invertebrate physiology, 2) examining anthropogenic impacts on aquatic ecosystems, and 3) developing tools for selecting oyster families with responses associated with improved performance. Federal agencies interested in these areas include NSF, NOAA, and USDA, respectively. In summary, funding via the Royalty Research Fund would allow my program to move into a new area of research that would complement my prior research activities and lay the groundwork for studying environmental physiology from an exciting new perspective.

Budget

01 Salary	
Steven Roberts, Assistant Professor (1 month summer salary)	8,040
Research Assistant, 50%, $1848 per month, 6 months, Fall 2009 and Winter 2010	11,088

03 Other Contractual Services	
UW Mass Spectrometry Center (analysis x 26) Type VIII	6,500

04 Travel	
Travel	300

05 Supplies and Materials	
Protein supplies: Extraction, Sample Preparation	1,500
RNA extraction reagents, qRT-PCR kits	1,500
general consumables (eg, plastics, tips, gloves, sample tubes, etc)	1,000

07 Retirement and Benefits	
01-10 Assistant Professor (24.1%)	1,938
01-40 Research Assistant (13.3%)	1,475

08 Operating Fee/Tuition	
08-05 Research Assistant, Two quarters, 2009 rate	6,222

TOTAL BUDGET 39,562

Budget Justification

Salary

Funds are requested for one month of summer salary for Assistant Professor Steven Roberts. In addition to developing the experimental approaches, he will spend 1 month carrying out the initial oyster treatments, compiling genomic resources for peptide comparison, and protein expression analysis. Funds are requested for 2 months of graduate student support. This Research Assistant will incorporate this into their thesis research and responsibilities will include oyster treatments, sample preparation, and gene expression analysis.

Budget Justification continued

Other Contractual Services

For identification of the proteins, samples will be submitted to the University of Washington's Mass Spectrometry Center for peptide analysis via LC-MS/MS. Analysis includes the protolytic digest a protein sample subjected to separation on a capillary LC coupled to the nano-spray source of a tandem hybrid mass spectrometer. The services include matching the digest's chromatogram against those of candidate proteins found in a database, with a probability scoring. This is classified as Type VIII analysis and cost $250 a sample.

Travel

$300 is requested to partially cover travel costs associated with sampling oysters and presenting research at local meetings.

Supplies and Materials

A total of $4000 is requested for materials and supplies to carry out the research objectives described as part of this project. $1500 is requested to cover cost of carrying out the oyster experiments, protein extraction, and sample preparation. We plan to submit 25 samples for analysis to the University of Washington's Mass Spectrometry Center. $1500 is requested to cover cost associated with objective 3 of the current project, examining the relationship of protein and gene expression. These funds will be used for reagents necessary to clone transcripts of corresponding proteins that have not been previously identified (ie degenerative primers, RACE kits). In addition, quantitative RT PCR will be carried out on samples stored from the initial stress treatments. Funds ($1000) are also requested to cover the common supplies and consumables used in work of this nature (i.e., gloves, pipettor tips, sample vials, PCR plates, etc)

CURRICULUM VITAE – STEVEN BEYER ROBERTS

Contact Information University of Washington
School of Aquatic and Fishery Sciences
Fisheries Teaching and Research Building
1140 NE Boat Street | Seattle, WA 98195
phone: 206.600.4495 | email: sr320@u.washington.edu
web: fish.washington.edu/genefish

Academic Experience Ph.D. – University of Notre Dame (South Bend, IN) – 2002
Integrative Cell and Molecular Physiology

B.S. – North Carolina State University (Raleigh, NC) – 1997
Natural Resources – Concentration in Marine and Coastal Resources

Professional Experience *2006-Present* · Assistant Professor
University of Washington, Seattle, WA

2006-Present · Adjunct Assistant Scientist
Marine Biological Laboratory, Woods Hole, MA

2003-2006 · Assistant Research Scientist
Marine Biological Laboratory, Woods Hole, MA

Select Publications

Roberts SB, Goetz G, White S, Goetz F (2008) Analysis of genes isolated from plated hemocytes of the Pacific Oyster, *Crassostreas gigas.* Marine Biotechnology. Jul 12. [Epub ahead of print]

Roberts SB, Gueguen Y, de Lorgeril J, Goetz F. (2008) Rapid accumulation of an interleukin 17 homolog transcript in *Crassostrea gigas* hemocytes following bacterial exposure. Developmental and Comparative Immunology. Volume 32, Issue 9, Pages 1099-1104

Roberts, SB (2008) USDA-NRAC Technical Report: "Development of genetic markers to assess disease resistance in the eastern oyster" 29 pages URL: http://tinyurl.com/23c7my

Lyons MM*, Lau Y-T, Carden WE, Ward JE, Roberts SB, Smolowitz R, Vallino J, Allam B. (2007) Characteristics of marine aggregates in shallow-water ecosystems: Implications for disease ecology. EcoHealth. 4, 406–420

Hodgins-Davis A*, Roberts SB, Cowan D, Atema J, Avolio C, Defaveri J, Gerlach G. (2007) Characterization of SSRs from the American lobster, *Homarus americanus.* Molecular Ecology Notes. 7:330-332

Rodgers BD, Roalson EH, Weber GM, Roberts SB, Goetz FW. (2007) A Proposed Nomenclature Consensus for the Myostatin Gene Family. AJP- Endocrinology and Metabolism. 292(2):E371-2

Lyons MM*, Smolowitz R, Dungan C, Roberts SB. (2006) Development of a real-time quantitative PCR assay for the hard clam pathogen, Quahog Parasite Unknown (QPX). Diseases of Aquatic Organisms. 72(1):45-52

Select Publications continued

Roberts SB, Romano C, Gerlach G. (2005) Characterization of EST derived SSRs from the bay scallop, *Argopectens irradians.* Molecular Ecology Notes. 5: 567-568

Biga PR, Roberts SB, Iliev DB, McCauley LA, Moon JS, Collodi P, Goetz FW. (2005) The isolation, characterization, and expression of a novel GDF11 gene and a second myostatin form in zebrafish, Danio rerio. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 141: 218-230

Roberts SB, McCauley LAR, Devlin RH, Goetz FW. (2004) Transgenic salmon over-expressing growth hormone exhibit decreased myostatin transcript and protein expression. Journal of Experimental Biology. 207(Pt 21):3741-8

Kim H-W*, Mykles DL, Goetz FW, Roberts SB. (2004) Characterization of an invertebrate myostatin homologue from the bay scallop, *Argopecten irradians.* BBA – Gene Structure and Expression. 1679(2):174-9

* *indicates student author*

Recent Presentations

Characterizing the response of *Vibrio tubiashii* to changes in environment. Pacific Coast Division, National Shellfisheries Association Conference, October 1-3, 2008. Chelan, WA

Overview of Shellfish Activities at the University of Washington. USDA-WERA099: Broodstock Management, Genetics and Breeding Programs for Molluscan Shellfish. April 6, 2008. Providence, RI

Characterizing disease resistance in native oysters that have experienced disease pressure National Shellfisheries Association Conference. April 6-10, 2008. Providence, RI

Disease tolerance and immune response in oysters. WA Resource Agencies: WA Department of Fish and Wildlife, WA Department of Natural Resources, and WA Department of Ecology. Olympia, WA. March 26, 2008

Immune response in shellfish. USGS Western Fisheries Research Center, Seattle WA. March 4, 2008

Gene expression profiling and cellular characteristics of *Crassostrea virginica* hemocytes: evaluating interactions of physical stress and disease. Aquaculture 2007, San Antonio, TX March 1, 2007

Genomic approaches in characterizing shellfish disease: interrelationships between animal, human and ecosystem health. Cummings School of Veterinary Medicine at Tufts University, Annual Symposium: Marine and Aquatic Medicine & Conservation. North Grafton, MA. April 22, 2006

Courses Taught

FISH310: Biology of Shellfishes
FISH507: Bioinformatic Approaches in Fisheries Science
FISH510: Innovations in Molecular Techniques
FISH414: Integrative Environmental Physiology (Winter 09)

Current Research Support - Roberts
Title: Evaluation of putatively QPX-resistant strains of northern hard clams using field and genetic studies
Role: Co-PI
Source of Support: USDA / Northeastern Regional Aquaculture Center
Award Amount: $70,490
Award Period: 03/01/2008 – 12/31/2010
Relationship: Characterizing stress / immune response at the transcript level in clams exposed to a pathogen. Clam samples are from field sites where environmental factors (ie temperature) are also considered.

Title: Development of tools to monitor and predict outbreaks of *Vibrio tubiashii*
Source of Support: School of Aquatic and Fishery Sciences, UW
Role: Co-PI
Award Amount: $24,644
Award Period: 08/01/08-07/30/09
Relationship: Characterizing virulence factors at the transcript level in *Vibrio* species that will also be used in the current proposal.

Past Research Support - Roberts
Title: Production of myostatin gene knockouts in zebrafish and the effects of specific myostatin interacting proteins
Role: Co-PI
Source of Support: USDA - NRI
Award Amount: $195,862
Award Period: 1/1/2005 - 11/30/2008
Relationship: Limited. Similar protein extraction and isolation methods are used.

Title: Assessing withering syndrome resistance in California Black Abalone: Implications for conservation and restoration
Role: Co-PI
Source of Support: California Sea Grant
Award Amount: $15,067
Award Period: 6/1/2007 - 5/30/2008
Relationship: Characterizing stress / immune response at the transcript level in abalone exposed to a pathogen. Abalone are in a controlled environment, the FSH holding facility.

Title: Development of genetic markers to assess disease resistance in the eastern oyster
Role: PI
Source of Support: USDA / Northeastern Regional Aquaculture Center
Award Amount: $154,066
Award Period: 9/1/2006-1/30/2008
Relationship: This project has the greatest relationship with the current proposal. During this project the physiological response (via gene expression level) was examine in wild and cultured oysters, from various locations, in relation to pathogen exposure and mechanical stress.

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Einarsdóttir IE, Anjos L, Hildahl J, Björnsson BT, Power DM. (2007) Isolation of Atlantic halibut pituitary hormones by continuous-elution electrophoresis followed by fingerprint identification, and assessment of growth hormone content during larval development. *Gen Comp Endocrinol* **150**(2).

Ferraro SP, Cole FA. (2004) Optimum benthic macrofaunal sampling protocol for detecting differences between four habitats in Willapa Bay, Washington. *Estuaries* **27**: 1014-25.

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Gourdine JP, Smith-Ravin EJ. (2007) Analysis of a cDNA-derived sequence of a novel mannose-binding lectin, codakine, from the tropical clam *Codakia orbicularis*. *Fish Shellfish Immunol.* **22**(5): 498-509.

Gueguen Y, Herpin A, Aumelas A, Garnier J, Fievet J, Escoubas JM, Bulet P, Gonzalez M, Lelong C, Favrel P, Bachère E. (2006) Characterization of a defensin from the oyster Crassostrea gigas. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. *J Biol Chem.* **281**(1):313-23.

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